OSMOTIC PROPERTIES OF THE ERYTHROCYTE

V. The Rate of Hemolysis in Hypotonic Solutions of Electrolytes

M. H. JACOBS AND ARTHUR K. PARPART

(From the Department of Physiology, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I

In an earlier paper in this series (Jacobs, 1932) it has been shown that, on the assumption that the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws is the factor of chief importance in determining the rate of osmotic hemolysis, the theoretical relation between the time at which some given degree of hemolysis is attained and the osmolar concentration of the surrounding solution ought to be given by the equation:

$$t_h = \frac{1}{k} \cdot \frac{V_0}{A} \left(\frac{c_0}{C^2} \ln \frac{c_0 - C}{c_0 - CR} - \frac{R - 1}{C} \right) \tag{1}$$

or, if the external medium be water alone, by

$$t_h = \frac{1}{k} \frac{V_0}{2c_0 A} (R^2 - 1), \tag{2}$$

where c_0 is the osmolar concentration of the solution in osmotic equilibrium with the normal erythrocyte, R the ratio of this concentration to that which will just cause the given degree of hemolysis, C the osmolar concentration of the external medium, V_0 the initial effective osmotic volume of the cell, A its area (assumed to be constant—a not unreasonable assumption in the case of the biconcave erythrocyte) and k the permeability constant of the erythrocyte for water; that is, a numerical measure of the amount of water that would with unit difference in osmotic pressure between the cell and its surroundings pass through unit surface in unit of time. Since V_0 and A are frequently not accurately known separately, the expression kA/V_0 may for many purposes be used as a secondary constant, k', whose calculated values over a range of concentrations give indication in the same way as do those of k of the applicability of the equations in question.

In the case of hypotonic solutions of non-electrolytes, it has already been shown (Jacobs, 1932) that the observed times of hemolysis over a wide range of concentrations are in fairly good agreement with those predicted by means of the equations, if allowance be made for a decided increase in the "osmotic resistance" of the cells produced by exposure to such solutions. Since there is some reason to believe that this increase in resistance may itself be osmotic in nature, there is no need at present to postulate non-osmotic factors to account fairly well for the observed results with non-electrolytes. In the case of electrolytes, however, which will be discussed in the present paper, conditions are somewhat different. In passing from water through a series of hypotonic solutions of, for example, NaCl of increasing concentration, the properties of the erythrocyte undergo a change, expressed quantitatively by a change in the value of the calculated permeability constant, which seems to depend on other than osmotic factors. Above a certain concentration—roughly 0.02M in the case of NaCl—the behavior of the erythrocyte is in excellent agreement with that predicted by means of the equations; that is to say, a constant calculated value of k' is obtained. Below this point, however, there is a fairly rapid increase in the value of k' with decreasing concentration which ceases only at very great dilutions of the electrolyte. This inconstancy of k', which almost certainly depends upon non-osmotic factors, and which is influenced to a striking extent by the valence of the cations present in the solution, has been very briefly mentioned in a previous preliminary paper (Jacobs, 1930) but has not hitherto been discussed at any length. We believe that it is of possible significance not only in connection with the problem of hemolysis but with certain larger ones having to do with the general question of cell permeability as well.

In the experiments here described, as well as in others omitted for lack of space, the blood used was that of the ox, obtained from freshly slaughtered animals, defibrinated immediately, and kept until needed in a refrigerator. A smaller number of experiments on the blood of man and of several other mammals gave essentially similar results. All observations were made at 20° C. $\pm$ 0.2° with the employment of exactly the same technique as that already described in the fourth paper of the present series (Jacobs, 1932), which may be consulted for further details.

II

In Fig. 1 are presented the results obtained on the same sample of blood with sucrose on the one hand and with NaCl on the other. In order that the results may be strictly comparable osmotically, the observed times of hemolysis are plotted as ordinates, not against the

concentrations of the two solutions, but rather against their freezing point depressions. The latter were calculated by the empirical equation for NaCl:

$$\triangle = 3.6C - 1.3C^2$$

and for sucrose

$$\triangle = 1.86C + 0.2C^2,$$

which for the concentration range actually employed give a fairly satisfactory agreement with published freezing point data.

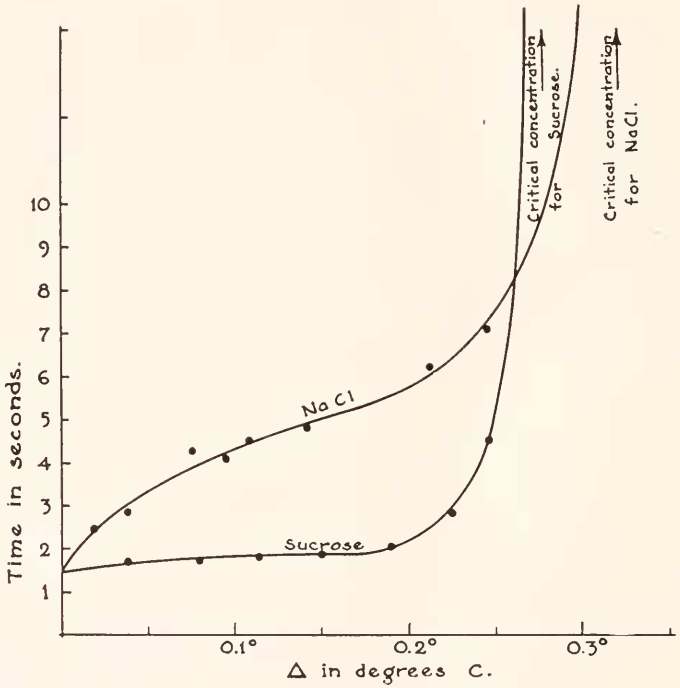

FIG. 1. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of sucrose and of NaCl. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ calculated freezing point depressions of solutions.

Two things are immediately apparent from the figure. The first is that over most of the range covered by the experiments hemolysis occurs far more slowly in NaCl solutions than in those of sugar of the same osmotic pressure. This difference is especially striking in the most dilute solutions (e.g., of $\triangle = 0.1°$ or less) where the osmotic effect of the solute as calculated by equation 1 is almost negligible, and where the effect actually observed with non-electrolytes is equally insignificant.

but where that found with electrolytes is very pronounced. As will be shown later, this electrolyte effect, which is exerted on the rate of hemolysis rather than on the position of final equilibrium of the system, is especially marked when the valence of the cations present is greater than one.

The second difference between the two curves to which reference has been made briefly above and at greater length in the preceding paper (Jacobs, 1932) is the lower critical hemolytic concentration, *i.e.,* the higher osmotic resistance of the cells, in the case of the non-electrolyte solution. In this particular case the value of Δ for which 75 per cent hemolysis just failed to occur was 0.324° for NaCl and 0.280° for sugar. This effect, which is of the "equilibrium" type, is obviously in the opposite direction from that of the first or "rate" effect, since electrolytes within the range where it is operative tend to favor rather than to oppose hemolysis. Because of the different natures of the two effects, the curves in Fig. 1 cross at a Δ value of about 0.26° for which the time of hemolysis is equal in the two solutions. Above this point there is a relatively narrow concentration range within which hemolysis actually occurs more rapidly in the presence than in the absence of the non-electrolyte. It is to be noted, therefore, that the observed rate of the hemolytic process may be affected by a mere shift in the position of final equilibrium of the system. Similar cases have been discussed elsewhere by the authors (Jacobs, 1928, 1931; Jacobs and Parpart, 1932).

The curve for NaCl in Fig. 1 shows very clearly the general relation between the concentration of a typical electrolyte solution and the time required for it to produce hemolysis; but for a more exact analysis of the extent to which such results are in agreement with osmotic laws it is necessary to employ more strictly mathematical methods. In Table I there have, therefore, been calculated by means of equations 1 and 2 for experiments with NaCl involving three separate samples of blood, values of the constant k', whose meaning is explained above and whose constancy over a given range may be taken as an indication of the applicability for this range of simple osmotic laws. The value of R employed for the calculations in each case was taken as the ratio of the freezing point depression of ox plasma (approximately 0.58° C.) to the freezing point depression of the NaCl solution in which, for the blood in question, the final degree of hemolysis was 75 per cent; this critical hemolytic concentration being determined for each sample of blood by a separate experiment. It was mentioned in the previous paper that a greater constancy of k' is obtained with non-electrolyte solutions if a somewhat smaller value of R than this be employed; but the theoretical justification for this latter procedure is rather questionable, and in the

calculation in that paper of the true permeability constant, k, the same R was used as that here adopted. It should be emphasized that in view of the complexity of the material and of the various simplifying assumptions made in deriving the equations a perfect agreement between theory and observation is never to be expected. For the present, therefore, it seems advisable to use the value of R which is most simply defined and most easily determined, even though a slightly different value may fit the data rather better in some particular cases.

TABLE I

Effect of the concentration of NaCl solutions on the time required for 75 per cent hemolysis of ox blood at 20° C. One part of blood to approximately 500 parts of solution. Each time is the average of four determinations.

Concentration	Δ	Experiment 1 $R = 1.63$		Experiment 2 $R = 1.79$		Experiment 3 $R = 1.69$	
		Time *seconds*	k'	Time *seconds*	k'	Time *seconds*	k'
0.00	0.000	1.35	1.06	1.42	1.34	1.30	1.23
0.005	0.018	2.48	0.62	3.00	0.64	2.28	0.73
0.01	0.036	2.72	0.58	3.75	0.55	2.68	0.64
0.02	0.072	3.60	0.48	4.82	0.48	3.78	0.51
0.03	0.107	4.70	0.41	5.65	0.46	4.68	0.46
0.04	0.142	5.12	0.42	6.15	0.48	5.05	0.48
0.05	0.177	5.65	0.43	6.88	0.49	5.60	0.50
0.06	0.211	6.40	0.44	7.85	0.52	6.68	0.48
0.07	0.246	7.80	0.44	10.62	0.47	7.48	0.53
0.08	0.280	11.02	0.39	35.90	0.20	10.78	0.48
0.09	0.314	30.22	0.20	—	—	130.	0.06

It will be noted in Table I that the value of k' for water alone in all three experiments is relatively high, *i.e.*, 1.06, 1.34, and 1.23, respectively. These values may be compared with those of 1.16 to 1.48 found in the previous paper, when R was similarly determined, for water and for a wide range of concentrations of sugar solutions. It will be further noted that whereas with the non-electrolyte discussed in the earlier paper no appreciable change in k' occurred in passing from water to solutions of a concentration of, say, 0.04M, in the case of NaCl, an enormous change appears on passing to a concentration of only 0.005M; and a further, though much slighter, change by an additional increase in the concentration to 0.01M. Even allowing for the fact that the osmotic pressure of an NaCl solution may be twice as great as that of a sugar solution of the same concentration, it is evident that the striking retardation of hemolysis caused by very dilute solutions of NaCl can scarcely be osmotic in nature.

Passing over the narrow range of concentrations from zero to 0.01M or 0.02M, within which k' undergoes a considerable change in magnitude, we find that for all higher concentrations up to 0.07M or 0.08M the value of k' is not only remarkably constant for a given experiment but that the values obtained with different samples of blood are in good quantitative agreement. It is difficult to believe that the constancy of k' over such a wide range of concentrations is due merely to chance. The most reasonable interpretation of the facts is that within this extensive range the concentration of an NaCl solution is related to the time of hemolysis by simple osmotic laws, as has already been found to be the case (with certain limitations) with non-electrolyte solutions. It is to be noted, however, that the value of the constant for NaCl solutions is only between one-half and one-third as great as for water and for non-electrolyte solutions. The same relation holds for the true permeability constant, k, which, for a given type of blood, is always a definite multiple of k'.

As to the complete lack of agreement between the last value of k' in each series with the remainder, it may be said that determinations of rates of hemolysis in solutions lying so close to the critical hemolytic concentration are notoriously unreliable, as has been pointed out by one of the authors elsewhere (Jacobs, 1928). Successive determinations under such conditions, even when carefully made, show such relatively enormous differences as to render exact quantitative work in this region almost hopeless. It is not unlikely that the very low values of k' at the highest concentrations, where the time of hemolysis exceeds about 10 seconds, may be significant, possibly indicating an escape of salts from the cell with a consequent retardation of hemolysis (see in this connection Ponder and Saslow, 1931); but in view of the difficulty of obtaining accurate data under these conditions we prefer to leave this point unsettled for the present. The important fact remains, nevertheless, that over a wide range the effect of the concentration of NaCl solutions on the rate of hemolysis is in good agreement with that demanded by simple osmotic laws.

III

Turning now to the region of the lowest concentrations (*i.e.*, all below about 0.02M), it is apparent that in this region small changes in the concentration of the electrolyte solution affect the rate of hemolysis in a manner that is not at all in agreement with equations 1 and 2. As a matter of fact, such effects extend to much more dilute solutions than any included in Table I and are, as will now be shown, intimately related to the valence of the cations present.

In Fig. 2 are presented the results of a typical experiment with a single sample of blood in which the time of hemolysis was determined in various hypotonic solutions of NaCl, Na_2SO_4, $CaCl_2$, $MgCl_2$ and $MgSO_4$. Since over most of the range employed osmotic effects must obviously be very slight, actual concentrations rather than freezing point depressions are used in the figure as abscissæ. Furthermore, in order that a wide range of concentrations, including those of a number of extremely dilute solutions, may be covered, the concentrations are

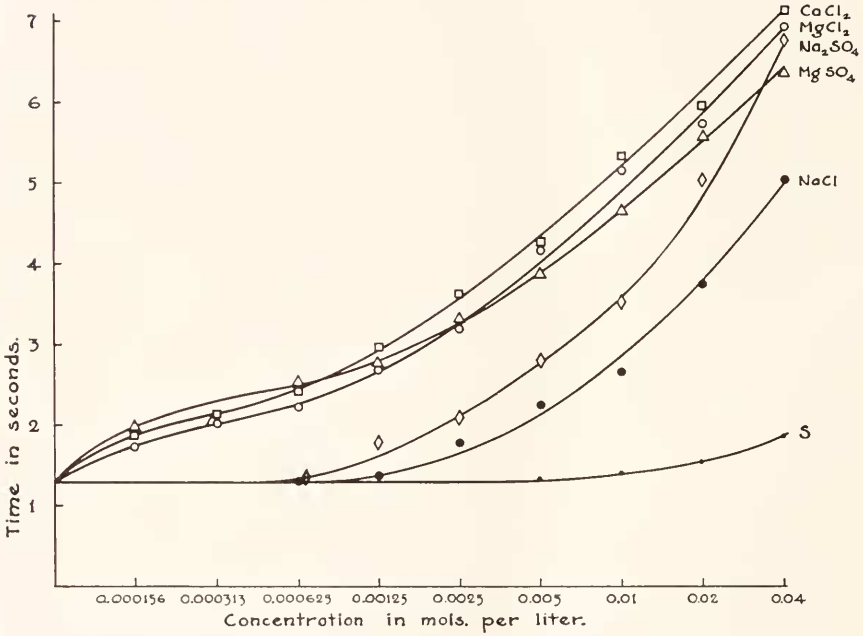

FIG. 2. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of various salts. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ concentrations of solutions in mols per liter.

plotted logarithmically, *i.e.*, equal distances along the axis of abscissæ are taken to represent equal multiples of concentrations rather than equal arithmetical increments. The figure is therefore comparable with those of Loeb (1922) to which reference will be made below. Included in the figure for comparison is a curve, labeled *S*, which indicates the calculated, and also approximately the observed, effects of sugar solutions having the osmotic pressures of the indicated concentrations of NaCl. It will be noted that the true osmotic effects, which alone are found in such solutions, are entirely negligible over most of the range

covered by the figure and that most of the effects of the electrolyte solutions must therefore be of a different nature.

An inspection of Fig. 2 brings out several additional points of interest. The first is that the salts fall into two sharply-separated groups, both with respect to the concentration at which a visible retardation of hemolysis first appears and with respect to the magnitude of the retardation at any given concentration. Thus, with $CaCl_2$, $MgCl_2$ and $MgSO_4$ a retardation of the order of 0.5 second or 40 per cent is present at a concentration of 0.00015M. A similar retardation is not reached with NaCl and Na_2SO_4 below a concentration of approximately 0.003M, and no detectable effect of any sort is found with either of the latter salts, or with KCl, which was studied in other experiments, below a concentration of about 0.001M. Throughout the entire range employed the relatively greater effectiveness of the salts of Ca and Mg is most marked. With salts of this type the valence of the cation appears to be the factor of chief importance, since there is little difference between $MgCl_2$ and $MgSO_4$.

In the case of NaCl and Na_2SO_4, both of which are rather widely separated in their properties from the salts just mentioned, it would appear that Na_2SO_4 is considerably more effective at a given concentration than is NaCl. This difference is probably to be attributed to the fact that the salt of the dibasic acid furnishes twice as many cations as that of the monobasic acid, the cation being, as already indicated, the ion of chief effectiveness in influencing the rate of hemolysis. If in plotting the two curves the concentrations of the Na ions had been used as abscissæ rather than the molecular concentrations, the curve for Na_2SO_4 would have been shifted to the right by an amount equal to that between two successive indicated concentrations; and in that case the two curves would have almost coincided. In several other experiments, not described here, the times of hemolysis for Na_2SO_4 in the region below 0.01M where osmotic effects are negligible were found to be somewhat below those for NaCl at the same $Na^{\cdot}$ ion concentration. In other words, with the same concentration of $Na^{\cdot}$, SO_4'' at times seemed, if anything, to favor hemolysis as compared with Cl', though it is to be noted that the concentrations of the two anions under these conditions were no longer the same and the differences were at best slight.

In the case of trivalent cations, a number of experiments have been made with $Al^{\cdot\cdot\cdot}$, but the results are too complex to be discussed here, since they involve $H^{\cdot}$ ion effects, agglutination of the erythrocytes, and other complications that have little bearing on the present problem. It may be mentioned, however, that in its ability to retard hemolysis at

very low concentrations, $Al^{\cdots}$, under proper conditions, may very considerably exceed the bivalent ions. With it a distinct retardation of hemolysis is at times obtained at concentrations as low as 0.00001M. The rather complicated nature of the effects of Al salts upon the erythrocyte will be discussed in detail elsewhere.

In addition to the experiments here described, a considerable number of others of the same general type have been performed. Because of the great rapidity of the hemolytic process in water and very dilute solutions, the quantitative accuracy of such experiments is not always as great as might be desired, and there are some slight discrepancies from experiment to experiment; but, on the whole, the results are in very satisfactory agreement and bear out the conclusion here reached, namely, that in dilute solutions cations tend to retard osmotic hemolysis in some non-osmotic manner with an effectiveness that increases greatly with an increase of their valence from one to two, and that anions have comparatively little influence on the process, though in some cases they seem with increasing valence slightly to favor it.

IV

As to the cause of the retardation of hemolysis produced by adding to distilled water electrolytes in concentrations from 0.01M to 0.0001M or even lower, it may be said with a fair degree of certainty that the osmotic pressure of the external solution in such cases is a factor of little or no significance. This is indicated not only by the negligible osmotic effects of such solutions as calculated by means of equation 1, and as actually observed in the case of non-electrolytes, but by the enormous differences in the effectiveness of, for example, NaCl and $MgSO_4$ at the same concentration, or of NaCl and $CaCl_2$ at the same freezing point.

The possibility nevertheless suggests itself that while in such cases the external osmotic pressure is of no importance, there might conceivably be produced by the solutions some indirect osmotic effects on the cells themselves which would influence the rate of the hemolytic process. We have already pointed out (Jacobs and Parpart, 1931) that the erythrocyte is unique among cells in the readiness with which its internal osmotic pressure is affected by apparently insignificant external changes of different sorts. Unfortunately for this explanation, such effects as might conceivably be produced in this way are, in the present case, in the wrong direction. As shown by the difference in the critical hemolytic concentration for electrolytes and for non-electrolytes (see Fig. 1), the "equilibrium" effect of electrolyte solutions is in the direction of favoring rather than of opposing hemolysis. An osmotic ex-

planation of the observed results, either direct or indirect, seems therefore definitely to be ruled out.

A more plausible explanation, because it suggests analogies in both living and in non-living systems, is that the rate of entrance of water into the erythrocyte is affected by low concentrations of ions in a manner similar to that observed by Lucké and McCutcheon (1929) in the case of the *Arbacia* egg and by Loeb (1922) in the case of collodion-gelatin membranes on the alkaline side of the isoelectric point of the gelatin. The former workers have reported that cations inhibit the passage of water into the *Arbacia* egg to an extent which increases with their valence, while anions behave in the opposite manner. In the case of collodion-gelatin membranes, where the factors concerned are obviously of a very simple physico-chemical nature, the results obtained are much the same; the nature of these effects has been discussed at length by Loeb. The erythrocyte differs from both the *Arbacia* egg and the artificial membrane in the much less prominent, and indeed somewhat doubtful, effect upon it of anions as compared with cations; but the striking difference between the ions of the alkali metals, on the one hand, and those of the alkaline earths on the other is found in all three cases, and may conceivably be due to the same causes.

An alternative explanation is that the effect of ions is on the rate of escape of hemoglobin from the cell rather than on the rate of entrance of water into it (see in this connection the discussion by Jacobs and Parpart, 1932, of the effect of narcotics on hemolysis). This explanation, however, while not completely ruled out by the existing evidence, seems to us to be less probable than the other one in view of the fact that the "equilibrium" effect of electrolytes on hemolysis, unlike that of narcotics, is in the opposite direction from the "rate" effect. Whatever the explanation of the effect of traces of electrolytes on the rate of hemolysis may ultimately prove to be, however, the observed facts are themselves entirely definite; and the non-osmotic factors shown to be concerned in the process would seem to be worthy of consideration in connection with theoretical discussions of the nature of cell permeability.

Summary

1. In NaCl solutions of concentrations from about 0.02M to 0.07M or 0.08M the rate of hemolysis of ox blood is related to the concentration of the solution as if the process were governed by simple osmotic laws.

2. The permeability constant for water over this range is between one-half and one-third as great as that previously found for non-electro-

lyte solutions. At concentrations below 0.02M the calculated "constant" changes with the concentration of the solution in a manner indicative of the presence of non-osmotic factors of some sort.

3. The retarding effect upon hemolysis of dilute solutions of electrolytes increases rapidly with the valence of the cations present. The valence of the anions is much less important but, if anything, acts in the opposite sense.

4. The tentative suggestion is offered that under certain conditions ionic forces may modify to an appreciable extent the rate of the osmotic intake of water by the erythrocyte.

BIBLIOGRAPHY

Jacobs, M. H., 1928. *Am. Nat.*, **62**: 289.
Jacobs, M. H., 1930. *Am. Jour. Med. Sci.*, **179**: 302.
Jacobs, M. H., 1931. *Ergebn. d. Biol.*, **7**: 1.
Jacobs, M. H., 1932. *Biol. Bull.*, **62**: 178.
Jacobs, M. H., and A. K. Parpart, 1931. *Biol. Bull.*, **60**: 95.
Jacobs, M. H., and A. K. Parpart, 1932. *Biol. Bull.*, **62**: 313.
Loeb, J., 1922. *Jour. Gen. Physiol.*, **4**: 463.
Lucké, B., and M. McCutcheon, 1929. *Jour. Gen. Physiol.*, **12**: 571.
Ponder, E., and G. Saslow, 1931. *Jour. Physiol.*, **73**: 267.